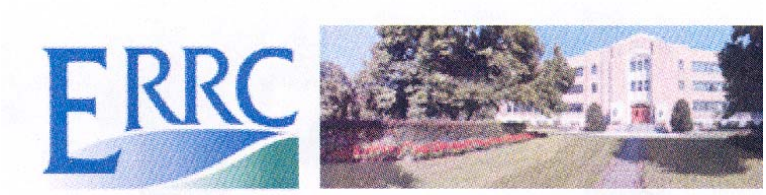

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Title: Factors Affecting the Efficacy of Chlorine Against Esherichia coli O157:H7 and Salmonella on Alfalfa Seed

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Factors affecting the efficacy of chlorine against *Escherichia coli* O157:H7 and *Salmonella* on alfalfa seed

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Factors affecting the efficacy of chlorine treatment for elimination of bacterial pathogens from laboratory-inoculated alfalfa seed, the ability of chlorine to eliminate Salmonella *Muenchen from naturally contaminated alfalfa seed, and the effect of chlorine treatment on germination of several types of sprouting seed were evaluated. Initial water temperature (15, 22 or 40°C) did not affect the rate or amount of free chlorine released from $Ca(OCl)_2$ during 30 min of stirring. Residual chlorine was detected after 15 min of stirring at the highest ratio of seed to volume of chlorine [50 g seed to 50 ml of 3% (w/v) $Ca(OCl)_2$] tested. Of the 29 types of sprouting seed treated for 10 min with buffered (to pH 6·8) 3·0% $Ca(OCl)_2$, only the germination of spelt and hard, soft and Kamut wheat was drastically reduced. Treatment (10 min) of laboratory-inoculated seed with buffered (to pH 6.8) 2·5 or 3·0% (w/v) $Ca(OCl)_2$ along with water rinses both before and after treatment led to a significant reduction (3·9–4·5 log cfu g^{-1}) of* Escherichia coli *O157:H7 and* Salmonella*, but not their elimination. Efficacy of chlorine treatment was reduced in the absence of buffer. Altering treatment times to 5 or 20 min did not affect treatment efficacy. A 1 h presoak of seed in tap water before chlorine treatment was beneficial, but led to a greatly reduced germination percentage after treatment. Addition of low levels of surfactant had no effect on treatment efficacy. Treatment (10 min with continuous agitation) of alfalfa seed naturally contaminated with Salmonella Muenchen with buffered or unbuffered 3·0% (w/v) $Ca(OCl)_2$, but not with buffered 0·3% (w/v) $Ca(OCl)_2$, eliminated the pathogen from the seed.*

Introduction

Many consumers in the US and in other countries eat sprouts in the raw state. Unfortunately, there have been numerous outbreaks of foodborne illness due to consumption of uncooked sprouts contaminated with human pathogens in several countries around the world (Mohle-Boetani et al. 2001, National Advisory Committee on Microbiological Criteria for Foods 1999, Taormina et al. 1999). Due to seed distribution systems some of these outbreaks have been international in scope. Alfalfa sprouts contaminated with various serotypes of *Salmonella* have been responsible for the majority of the US outbreaks, but two alfalfa sprout-related outbreaks were due to contamination with *E. coli* O157:H7 or O157:NM. The largest sprout-related outbreak ever recorded took place in 1996 in Japan with over 7000 illnesses due to consumption of Daikon (white) radish sprouts contaminated with *E. coli* O157:H7 (Michino et al. 1999). The

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earliest of the recorded US outbreaks took place in 1973 and was due to consumption of mixed sprouts grown from home sprouting kits containing seed contaminated with *Bacillus cereus* (Portnoy et al. 1976). The primary source of the contaminating human pathogens for most outbreaks is thought to be the seed used for sprouting. Sprouting seed is obtained from plants grown in open fields often without consideration that the seed may later be used for growing sprouts intended for human consumption (National Advisory Committee on Microbiological Criteria for Foods 1999). Seed as the origin of the bacterial pathogens is usually based on epidemiological evidence, but for some outbreaks the pathogen can be isolated from the implicated seed lot (Inami and Moler 1999).

In order to curtail sprout-related outbreaks, seed producers need to implement appropriate Good Agricultural Practices and sprout growers need to follow Good Manufacturing Practices. In addition, development of Hazard Analysis and Critical Control Point plans and Sanitation Standard Operating Procedures as well as third party audits are desirable. In October of 1999, the US Food and Drug Administration (FDA) issued two guidance documents to advise sprout growers and seed suppliers of steps to take to reduce the potential for contamination of sprouts with human pathogens (US FDA 1999). More recently, the US FDA and The State of California released a food safety training program video for use by sprout growers (Waddell and Troxell 2000). In these materials, as part of a comprehensive program to ensure safe sprouts, growers were advised of the need to employ an effective seed sanitization step using calcium hypochlorite providing approximately 20 000 ppm of free chlorine or another approved sanitizer with equivalent or greater efficacy as well as test the spent irrigation water for the presence of *Salmonella* and *E. coli* O157:H7. Rinsing of the seed with potable water both before and after the sanitizer treatment is also recommended. To date, the only chemical approved by the US Environmental Protection Agency for use on sprouting seed is free chlorine at a level up to 20 000 ppm. Several studies on the efficacy of chlorine and various other aqueous chemical sanitizers to sanitize alfalfa seed inoculated in the laboratory with *Salmonella* or *E. coli* O157:H7 have recently appeared in the literature (Beuchat 1997; Jaquette et al. 1996; Taormina and Beuchat 1999; Weissinger and Beuchat 2000; Lang et al. 2000; Beuchat et al. 2001). None of these studies employed seed rinses both before and after sanitizer treatment. While this manuscript was in preparation, Stewart et al. (2001) published the first report on the efficacy of treating naturally contaminated seed with 20 000 ppm of free chlorine.

The primary goals of this study were to determine: (1) the effect of the ratio of alfalfa seed to volume of chlorine solution and the duration of mixing on levels of residual free chlorine, (2) the effect of chlorine treatment on the germination on a wide variety of sprouting seed types, (3) the effect of chlorine treatment of seed in combination with seed rinses before and after treatment on the elimination of *E. coli* O157:H7 and *Salmonella* from laboratory-inoculated alfalfa seed and (4) the ability of chlorine treatment to eliminate *Salmonella* Muenchen from naturally contaminated alfalfa seed.

Materials and Methods

Bacterial strains

Escherichia coli O157:H7 strain F4546 (a clinical isolate associated with a sprout-related outbreak in Michigan and Virginia in 1997) was obtained from Dr Robert Buchanan, FDA, CFSAN, Washington, District of Columbia, USA. Strains C7927 (a clinical isolate associated with a apple cider-related outbreak in Massachusetts in 1992), SEA13B88 (originally isolated from apple cider implicated in an outbreak in the Western United States in 1996) and strain Ent-C9490 (a clinical isolate associated with a hamburger-related outbreak in the Western United States in 1993) were obtained from Dr Pina Fratamico, Eastern Regional Research Center, Wyndmoor, Pennsylvania, USA. *Salmonella* Anatum F4317, *Salmonella* Infantis F4319, *Salmonella* Newport H1275 and *Salmonella* Stanley H0558 were obtained from Dr Patricia Griffin, Center for Disease Control and Prevention, Atlanta, Georgia, USA. All four

strains of *Salmonella* were implicated in sprout-related outbreaks in the US. To check for inhibition among strains, the bacterial strains belonging to each genus were cross-streaked on tryptic soy agar (TSA) (Difco Laboratories, Detroit, Michigan, USA) and the plates incubated at 37°C for 24 h. No inhibition of bacterial growth was noted. For long-term storage, strains were suspended in tryptic soy broth (TSB) (Difco) supplemented with 20% glycerol and kept at −80°C.

Seed

Sprouting seed was obtained from commercial sources. For use in experiments designed to test the efficacy of chlorine treatments, seed to be inoculated was sterilized by exposure to gamma irradiation (25 kGy, cesium-137 source) at 20°C to eliminate native microflora. To confirm the effectiveness of the radiation treatment irradiated seed (10 g) was placed in a sterile stomacher bag, 40 ml of TSB was added and the bags placed at 30°C for 24 h. Undiluted samples were then plated on tryptic soy agar (TSA) (Difco) (0·25 ml per plate, four plates per sample) and on total aerobe and *E. coli*/coliform Petrifilm count plates (1·0 ml per plate, two plates per sample) (3M, St. Paul, Minnesota, USA). All plates were incubated at 35°C except for the total aerobe Petrifilm count plates (30°C).

Alfalfa seed lot COA98 that was naturally contaminated with *Salmonella* Muenchen was supplied by Dr Mary Lou Tortorello, US Food and Drug Administration, National Center for Food Safety and Technology, Summit Argo, Illinois, USA.

Method for determining levels of free chlorine

Free chlorine levels were determined with an EPA-approved commercial test kit (Accuvac®, Hach Company, Loveland, Colorado, USA) based on the *N,N*-diethyl-*p*-phenylenediamine (DPD) colorimetric method. Required sample dilutions were made with purified water (18·2 MΩ cm resistivity) before free chlorine determination. In order to eliminate any chlorine demand all glassware used to prepare sample dilutions was first presoaked in 52·5 ppm sodium hypochlorite (1:1000 dilution of commercial bleach) prepared in purified water for 90 min, rinsed well with purified water and then air-dried in an oven before use (Anonymous 1997).

Effect of time of stirring and tap water temperature on release of free chlorine from $Ca(OCl)_2$

To test the effect of the time of stirring and temperature of the suspending medium on release of free chlorine from $Ca(OCl)_2$ (Aldrich Chemical Company, Inc., Milwaukee, Wisconsin, USA) a 3% (w/v) solution was prepared in tap water at three different temperatures and continuously mechanically stirred for 30 min without temperature control. The initial temperatures of the tap water were 15°C (cold water from the tap), room temperature (22°C), or 40°C (hot water from the tap). At 5 min intervals up to a total of 30 min of stirring, samples were removed, diluted with purified water as required and free chlorine levels were determined as stated above. The experiment was done twice.

Effect of seed-to-volume ratio and time of stirring on the level of residual free chlorine

Calcium hypochlorite (3% w/v) was prepared in 50 ml of room temperature (22°C) sterile tap water with mechanical stirring for 20 min at room temperature before addition of alfalfa seed. Seed (10, 20, 30, 40 or 50 g) was then added, the suspension was continuously mechanically stirred and samples were removed after 5, 10, and 15 min and the free chlorine levels were determined as described above. The experiment was done twice.

Determination of the effect of chlorine treatment on sprouting seed germination

Seeds were placed in 3% (w/v) of $Ca(OCl)_2$ prepared in sterile 500 mM potassium phosphate buffer, pH 6.8 (hereafter referred to as KP buffer), at a weight-to-volume ratio of 1 g seed per 5 ml of solution. The seed/sanitizer mixture was stirred for 10 min on a magnetic stirrer. The treated seeds were rinsed twice with sterile

tap water (volume equal to that of the sanitizer solution), and then soaked in sterile tap water for a minimum of 2 h. Small seed types were then transferred to sterile glass culture dishes lined with two pieces of wetted filter paper (Whatman #2) with 100 seeds per dish. Large seed types were placed into sterile glass trays lined with wetted germination paper (Seedburo Equipment Company, Chicago, Illinois, USA) with 100 seeds per tray. Seed trays were covered with plastic wrap and both trays and culture dishes containing seed were kept at room temperature (20–22°C). Additional sterile tap water was added as required. A seed was recorded as germinated if a protruding radicle was visible to the naked eye. Germination percentage was recorded daily and each seed type was tested twice.

Inoculation of sterile alfalfa seed with bacterial human pathogens

To prepare an inoculum cocktail consisting of four strains of *E. coli* O157:H7, the strains were grown in TSB with 1% (w/v) dextrose (added an additional 7.5 g l^{-1}) for 18 h at 37°C without agitation. Strains of *Salmonella* were grown in TSB without additional glucose for 18 h at 37°C with shaking (250 rpm). After incubation, cultures (25 ml each) were combined and the cells pelleted by centrifugation. The pelleted cells were washed once with sterile 0·1% peptone-water (PW, Difco) and then resuspended in sterile PW to a final inoculum volume of 200 ml. Inoculum concentration was determined by dilution plating onto TSA. Inoculum concentrations for bacterial cocktails were approximately 5×10^8 cfu ml^{-1} and approximately 8×10^9 cfu ml^{-1} for *E. coli* O157:H7 and *Salmonella*, respectively. Fifty milliliters of inoculum was added to each of the four sterile filter stomacher bags containing 100 g of irradiated alfalfa seed. After massaging the bags by hand for 1 min, the excess inoculum was removed. The inoculated seed was then placed on a sterile screen, allowed to dry for 48 h under a biological safety cabinet and then stored until used at 4°C. The initial inoculum level on the seed was determined by addition of 10 g of inoculated seed to a sterile filter stomacher bag followed by addition of 20 ml of sterile PW. The bag was subjected to pummeling as stated above. Serial decimal dilutions were prepared in sterile PW. For enumeration of *E. coli* O157:H7, the dilutions were plated onto *E. coli*/coliform and *Enterobacteriaceae* Petrifilm count plates (1·0 ml per plate) (3M), Levine EMB agar (Difco) and TSA. For enumeration of *Salmonella*, dilutions were plated onto TSA and XLT-4 (Difco). Colonies were first counted after 24 h of incubation at 37°C and then recounted after 48 h.

Sanitizing laboratory-inoculated alfalfa seed with chlorine

Chlorine solutions were prepared by addition of $Ca(OCl)_2$ in varying amounts (0·15–1·50 g) to 50 ml of room temperature sterile KP buffer or sterile tap water and continuously mixing on a magnetic stir plate for 20 min at room temperature before use. The basic protocol for seed treatment was as follows: alfalfa seed (10 g) inoculated with *E. coli* O157:H7 or *Salmonella* as stated above was placed into a sterile stomacher bag. The seed was washed twice by addition of 50 ml sterile tap water followed by massaging the bags by hand for 2 min and decanting the wash fluid. After the second wash, 50 ml of chlorine solution or sterile tap water was added. The contents of the bag were massaged by hand for 5–20 min, the liquid was decanted and the seed washed twice with sterile tap water as stated above. Twenty milliliters of sterile buffered PW (Difco) was added and the bags were pummeled for 1 min at medium speed. Serial decimal dilutions were prepared in sterile PW and the dilutions plated in triplicate on selective (*E. coli*/coliform plates, 1·0 ml per plate; XLT-4, 0·1 ml per plate for *Salmonella*) and non-selective media (TSA, 0·1 ml per plate). The inoculated Petrifilm count plates were incubated at 37°C for 2 days before the final colony counts were obtained. Final colony counts for growth on TSA were determined after 24 h. The identity of at least two colonies per TSA plate selected at random was confirmed as *E. coli* O157:H7 or *Salmonella* by use of either latex agglutination (commercial kit RIM *E. coli* O157:H7; Remel, Lenexa, Kansas USA) or slide agglutination (Bacto *Salmonella* Poly A-I and Vi antiserum; Difco). Samples were

also enriched for survivors by addition of 20 ml of TSB to each stomacher bag after removal of initial samples for dilution plating, the bags incubated at 37°C for 24 hr and then four 0·25 ml samples were plated onto TSA with incubation at 37°C for 24 h. In addition, seed was treated with 500 mM CAPS buffer, pH 11·0 (Sigma Chemical Co., St. Louis, Missouri, USA). Controls consisted of seed treated for 10 min with sterile KP buffer or sterile tap water alone. Experiments were done two to four times.

Treatments with unbuffered 3% (w/v) $Ca(OCl)_2$ prepared in sterile tap water were also carried out with various surfactants added to the chlorine solutions immediately before treating the seed. The surfactants tested were Tween 80 (0·02%, v/v), SDS (sodium lauryl sulfate, 0·02%, w/v) (Sigma Chemical Company, St. Louis, Missouri, USA) and a commercial 'chlorine potentiator' (a mixture of emulsifiers, BonAgra CP-100, BonAgra Technologies & Services, Inc., Tucson, Arizona, USA) at a level of 0·084% (v/v) as recommended by the manufacturer. The experiment was done three times.

The effect of a 1 h presoak of seed in sterile tap water on the efficacy of the chlorine treatment was also evaluated. After the initial rinse with sterile tap water, the seed was left to soak for 1 h before the second rinse with sterile tap water. This was followed by treatment with chlorine and the additional rinses. The effect of the presoak step on germination of non-irradiated seed was determined as described above. The experiment was done four times.

Treatment of naturally contaminated seed with chlorine

Two protocols were used to determine the ability of free chlorine provided by $Ca(OCl)_2$ to eliminate *Salmonella* Muenchen from alfalfa seed lot COA98. For protocol A, three samples of naturally contaminated alfalfa seed (200 g each) were placed into 2 l sterile glass beakers and rinsed twice with 800 ml of sterile tap water with continuous stirring by hand using a sterile spatula for 2 min per rinse. The final rinse water was discarded, 800 ml of 3% (w/v) $Ca(OCl)_2$ prepared in 500 mM KP buffer, pH 6·8, was added and the seed continuously stirred for 10 min by hand. Three samples of seed treated with sterile buffer alone were used as the controls. The treated seeds were rinsed twice with 800 ml sterile tap water for 2 min and then transferred in sterile glass trays (27 cm × 38 cm × 5 cm) lined with wetted seed germination paper. The trays were covered with aluminum foil and the seeds allowed to germinate at room temperature (20–22°C) on a lab bench for 4 days with daily addition of sterile tap water (50–100 ml).

To determine the presence of *Salmonella* Muenchen on the sprouts, a total of 100 g of sprouts was harvested from nine evenly divided sectors in each tray. The sprouts were placed in a sterile stainless-steel blender receptacle and 500 ml of lactose broth (Difco) was added. The mixture was homogenized for 1 min using a commercial blender and the homogenate transferred to a filter-lined stomacher bag. Homogenization of sprouts in a blender rather than in a stomacher is recommended in the US FDA guidance documents (US FDA 1999). After sitting for 1 h at room temperature on the lab bench, the pH of the homogenate was adjusted to approximately 7·0 by addition of sterile 5 N NaOH followed by incubation at 37°C without shaking for 18–24 h. After incubation a 1 ml sample of the non-selective enrichment broth was transferred to 10 ml of tetrathionate (TT) broth (Difco) and 0·1 ml to 10 ml of Rappaport-Vassiliadis R10 (RV) broth (Difco) with four tubes for each selective broth medium. Incubation was at 37°C (TT broth) or 42°C (RV broth) for 18 h without shaking. After incubation, 50 µl samples from each tube were streaked onto single plates of Hektoen Enteric (HE) agar and XLT-4 agar (Difco) and the inoculated selective agar media placed at 37°C. The plates were examined after 24 and 48 h for the presence of black colonies typical of the presence of *Salmonella*. Suspect colonies were selected at random and purified by repeated streaking on XLT-4. Slide agglutination tests utilizing Bacto *Salmonella* O antiserum poly A-I and Vi (Difco) and biochemical testing with BBL Enterotube II multi-media tubes for rapid identification of *Enterobacteriaceae* (Becton Dickinson and Company, Cockeysville, Maryland, USA) done according to the manufacturer's instructions were used to confirm

isolates as *Salmonella*. For determination of serotype, *Salmonella* isolates were submitted to the APHIS National Veterinary Services Laboratory, Ames, Iowa, USA.

For protocol B, 100 g samples of seed were placed into 1 l glass beakers and rinsed twice with sterile tap water (200 ml per rinse) as described above. The seed was then treated with 0·3 or 3·0% (w/v) $Ca(OCl)_2$ solutions prepared in 500 mM KP buffer, pH. 6·8, or with 3·0% (w/v) $Ca(OCl)_2$ prepared in sterile tap water for 10 min with continuous agitation by hand using a sterile spatula. The treated seed was rinsed twice with sterile tap water as described above. Seeds treated with buffer (500 mM KP, pH 6·8) and sterile tap water alone were used as controls. Treated seeds were transferred to sterile glass jars (800 ml) (Stewart et al. 2001), 350 ml of sterile tap water was added and the seed left to soak for 3 h at room temperature (20–22°C). The soak water was removed and the seed was allowed to germinate at room temperature. The growing sprouts were rinsed with 250 ml of sterile tap water daily. On the third day, the sprouts were rinsed with 400 ml sterile tap water and the rinse water was analyzed for the presence of *Salmonella* Muenchen as described below. Also, 100 g of sprouts were homogenized in 500 ml of lactose broth and the homogenate used to analyse for *Salmonella* Muenchen as for Protocol A.

To test for the presence of *Salmonella* Muenchen in rinse water, 25 ml of rinse water per sample was added to 225 ml of buffered PW contained in 1 l Erlenmeyer flasks to which 4 ml of 0·1% (w/v) filter-sterilized novobiocin (Sigma Chemical Company, St. Louis, Missouri, USA) was added immediately before use. The rest of the procedure was as recommended in the US FDA guidance documents (US FDA 1999) for use of the Visual Immunoprecipitate (VIP™) assay for *Salmonella* (Bio-Control Systems, Inc., Bellevue, Washington, USA) (AOAC Official Method 1B 999.09). For positive immunoassay test results, the presence of *Salmonella* was confirmed by streaking from the tubes containing TSB+DNP+n onto selective agar media (HE and XLT-4). Purified suspect colonies were then subjected to serological and biochemical testing as described above. Selected isolates confirmed to be *Salmonella* were submitted to the APHIS Laboratory for serotyping.

Statistical analyses

Data on the effects of chlorine treatment on laboratory-inoculated seed was analysed for significant differences ($P<0·05$) by use of the least significant difference (LSD) separation procedure (Miller 1981). Statistical analysis for the data generated for the treatments of naturally contaminated seed with free chlorine was carried out to determine if the number of positive samples in the untreated controls were statistically non-zero. The analysis was done on arcsine-square root transformations of the binomial response with the value of $1/4n$ replacing the 0 values (Snedecor and Cochran 1989).

Results and Discussion

Effect of tap water temperature and duration of stirring on release of chlorine from calcium hypochlorite

The amount and rate of free chlorine released when 3·0% (w/v) $Ca(OCl)_2$ was suspended in three temperatures (15, 22 or 40°C) of tap water was determined. After 5 min of continuous mechanical stirring, 18 000 ppm of free chlorine was released under all conditions and this level remained constant over a total stirring time of 30 min.

Effect of ratio of seed to volume of sanitizer on residual free chlorine

For this set of experiments 3% (w/v) $Ca(OCl)_2$ was prepared in sterile tap water (18 000 ppm free chlorine). The ratios of seed to volume tested ranged from 10 to 50 g seed per 50 ml of chlorine solution with residual free chlorine measured after 5, 10 and 15 min of continuous stirring. Results indicated that the reduction in the levels of free chlorine over the 15 min period was dependent on the amount of seed present (Fig. 1). The lowest level of residual free chlorine in solution (approximately 100 ppm) was seen after mixing 50 g seed in 50 ml of chlorine solution for 15 min. With a ratio of

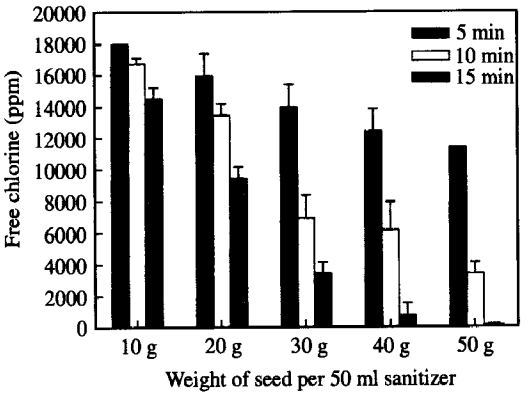

Figure 1. The effect of alfalfa seed weight on the residual free chlorine levels for three treatment times. Values shown are the means of data from two separate experiments. Error bars for each mean represent the standard deviation. Where no error bars are evident the standard deviation was zero; ■, 5 min; □, 10 min; □, 15 min.

10 g seed to 40 ml of chlorine solution and an initial free chlorine concentration of 20 000 ppm, Taormina and Beuchat (1999) found a 20% decrease in the amount of free chlorine remaining after a 15 min treatment period. Thus, free chlorine in solution remains at a level that will rapidly kill any pathogens washed from the seed even when seed-to-volume of sanitizer (18 000–20 000 ppm free chlorine) ratios are as high as 1:1 (wt/vol) over the recommended 15 min treatment period (US FDA 1999).

Effect of chlorine treatment on germination of sprouting seed

After a 10 min treatment with buffered 3% (w/v) $Ca(OCl)_2$ (16 000 ppm free chlorine), most seed types demonstrated delayed germination (data not shown). Percent germination values determined at 2 days for rapidly germinating seed or at 3–4 days for slower germinating seed are shown in Table 1. By this time most chlorine-treated seed types had germination percentages similar to the controls. Four seed types (hard wheat, soft wheat, Kamut wheat and spelt) showed significantly reduced germination. Garbanzo bean seed was not adversely affected in germination rate or percentage, but the roots were malformed. Treatment of blackeyed pea seed with chlorine substantially increased germination. The results for alfalfa seed correspond with reports from other laboratories (Lang et al. 2000; Taormina and Beuchat 1999; Weissinger and Beuchat 2000). The results indicate that high levels of chlorine may be used for sanitizing most sprouting seed types without adverse effects on germination.

Table 1. Effect of a chlorine treatment on the germination of sprouting seed*

	% Germination				% Germination		
Seed type	Day	Chlorine	Control	Seed type	Day	Chlorine	Control
Adzuki bean	3	99±1†	94±0	Lentil (green)	2	93±0	88±1
Alfalfa	2	94±1	96±2	Lentil (red)	2	46±2	32±6
Barley	3	69±5	56±2	Mung bean	2	97±4	98±1
Cow bean	2	74±2	71±6	Mustard cress	2	74±9	87±3
Blackeyed pea	3	90±7	36±2	Onion chive	4	72±3	77±6
Broccoli	2	88±2	92±5	Radish (Daikon)	2	98±3	97±1
Cabbage	2	98±2	99±1	Radish (China Rose)	2	88±6	86±1
Canola	2	100±0	98±1	Soybean	2	93±4	95±7
Cauliflower	2	74±4	83±1	Snow pea	2	69±4	63±16
Clover	2	92±2	98±1	Spelt	3	18±8	38±4
Fenugreek	2	92±1	86±1	Sunflower	2	86±2	82±1
Garbanzo bean	2	98±1	91±2	Wheat (hard)	2	31±8	94±1
Green pea	3	96±1	89±2	Wheat (soft)	2	27±1	87±1
Kale	2	78±11	89±11	Wheat (Kamut)	2	0±0	88±8
Leek	3	84±2	91±4				

*Seeds (100 seeds per experiment per type) were treated with 3·0% (w/v) $Ca(OCl)_2$ prepared in 500 mM KP buffer, pH 6·8 or KP buffer alone (controls) for 10 min with constant agitation.
†Values shown are means of data from two separate experiments ±s.d.

However, sprout quality, yield and/or shelf-life may be adversely impacted for certain types of sprouts. In addition, different lots of a particular sprouting seed type may differ in their ability to withstand treatment with high levels of chlorine.

Effectiveness of chlorine treatments for sanitizing laboratory-inoculated seed

Cells of *E. coli* O157:H7 were induced to pH-dependent, stationary phase acid resistance. Such stressed cells exhibit cross-resistance to a variety of environmental stresses and may more closely mimic *E. coli* O157:H7 in natural environments (Buchanan et al. 1999, Buchanan and Edelson 1999). For *E. coli* O157:H7, the inoculum concentration on the seed immediately following air-drying was approximately 6·5 log cfu g^{-1} based on plating on selective and non-selective agar media. For *Salmonella*, the bacterial populations on the seed immediately after air-drying averaged approximately 8·5 log cfu g^{-1} based on plating on TSA and 8·0 log cfu g^{-1} based on plating on the selective medium XLT-4. Thus, most bacterial cells present on the seed immediately after inoculation and air-drying appeared to be uninjured. Based on approximately 500 alfalfa seed g^{-1} and assuming an even distribution of bacterial cells per seed, the seed inoculated with *E. coli* O157:H7 harbored approximately 6×10^3 cfu per seed. For *Salmonella* the corresponding value was approximately 6×10^5 cfu per seed. The inoculated seed was used within 2 months of storage at 4°C.

For a related study in our laboratory, alfalfa seed that had been inoculated with the cocktail of the same strains of *E. coli* O157:H7 as used in this study (initial inoculum load = 5·30 $\log_{10}$ cfu g^{-1}) and stored for 30 months at 4°C showed a decrease in inoculum level of 0·88 $\log_{10}$ cfu g^{-1} For seed that had been inoculated with a cocktail of the same *Salmonella* strains (initial inoculum load = 5·70 or 3·85 $\log_{10}$ cfu g^{-1}) and stored for 36 months at 4°C, a decrease in inoculum level of 0·44 $\log_{10}$ cfu g^{-1} was found for the seed with the higher initial inoculum level and no reduction for the seed with the lower initial inoculum level. Thus, populations of *E. coli* O157:H7 and *Salmonella* on laboratory-inoculated alfalfa seed were quite stable when the seed was stored at 4°C. Lang et al. (2000) reported a decrease of $<0{\cdot}2$ $\log_{10}$ cfu g^{-1} in populations of *E. coli* O157:H7 on inoculated alfalfa seed after 8 weeks of storage at 5°C. However, in a longer-term study, Taormina and Beuchat (1999) found that populations of *E. coli* O157:H7 on inoculated alfalfa seed decreased by 3 $\log_{10}$ cfu g^{-1} after storage at 5°C for 54 weeks. Variability in the results for survival of *E. coli* O157:H7 on inoculated alfalfa seed stored at refrigeration temperatures may be due to strain differences and/or to differences in percent relative humidity during storage. For alfalfa seed inoculated with *Salmonella*, Weissinger and Beuchat (2000) reported no significant change in population on seed stored at 5°C for up to 6 weeks. Storage at higher temperatures appears to be more detrimental to pathogen survival. Taormina and Beuchat (1999) found a much more rapid decrease in the population of *E. coli* O157:H7 on inoculated alfalfa seed stored at 25°C or 37°C than at 5°C. Jaquette et al. (1996) reported that storage of seeds inoculated with *Salmonella* Stanley for 8–9 weeks at 8°C and 21°C led to reductions in populations of about 1 $\log_{10}$ and 2 $\log_{10}$ cfu g^{-1}, respectively. However, Inami et al. (2001) recently reported that *Salmonella* Newport was detected from a naturally contaminated seed lot after 2 years of storage at room temperature in the dark.

Initial experiments tested for the ability of water rinses plus 10 min chlorine treatments to eliminate *E. coli* O157:H7 or *Salmonella* (Table 2). A high level (500 mM) of KP buffer, pH 6·8, was required to maintain the pH of a 3% (w/v) solution of $Ca(OCl)_2$ below pH 7·0. Based on determination of free chlorine levels, this molarity of buffer appeared to reduce the amount of free chlorine present compared to similar solutions prepared in sterile tap water (16 000 ppm versus 18 000 ppm free chlorine, respectively). Rinsing along with 10 min treatments with sterile buffer or tap water alone led to a minimal reduction ($>0{\cdot}5$ log cfu g^{-1}) of *E. coli* O157:H7 on the seed (Table 2). Rinsing plus treatment with buffer alone removed a greater amount of *Salmonella* (1·9 log cfu g^{-1}) possibly due to the higher initial population on the seed. Initially, a comparison was made

Table 2. Effect of chlorine treatments on *E. coli* O157:H7 or *Salmonella* inoculated onto alfalfa seed

Treatment†	Solution pH	Time (min)	*E. coli* O17:H7 Survivors (log cfu g^{-1})* TSA‡	*E. coli* O17:H7 Survivors (log cfu g^{-1})* Petrifilm	*E. coli* O17:H7 Log cfu g^{-1} reduction§	*Salmonella* Survivors (log cfu g^{-1}) TSA	*Salmonella* Survivors (log cfu g^{-1}) XLT-4	*Salmonella* Log cfu g^{-1} reduction‡
Experiment 1:								
Buffer (500 mM KP)	6·8	10	6·58±0·02^{a}	6·44±0·10^{a}	—	6·60±0·51^{a}	5·68±0·52	—
Buffered chlorine								
2·0% (11 000 ppm)‖	6·8	10	4·96±1·15^{b}	4·18±1·60^{b}	1·62			
2·5% (13 500 ppm)	6·8	10	2·54±0·34^{c}	n.d.¶	4·04			
3·0% (16 000 ppm)	6·8	10	2·07±0·18^{c}	n.d.	4·51	2·69±0·20^{b}	n.d.	3·91
Experiment 2:								
Sterile tap water	6·7	10	6·34±0·10^{a}	6·02±0·12^{a}	—			
Chlorine in water								
2·0% (14 000 ppm)	11·4	10	4·90±0·06^{b}	4·38±0·08^{b}	1·44			
2·5% (16 000 ppm)	11·5	10	3·04±0·08^{c}	1·98±0·23^{c}	3·30			
3·0% (18 000 ppm)	11·6	10	2·89±0·08^{c}	1·82±0·00^{c}	3·45			
Experiment 3:								
Sterile tap water	6·7	10	6·13±0·01^{a}	5·59±0·40^{a}	—			
Buffer (500 mM CAPS)	11·0	10	5·45±0·23^{a}	5·23±0·30^{a}	0·68			
Experiment 4:								
Sterile tap water	6·7	20	6·10±0·41^{a}	5·87±0·38^{a}	—			
Chlorine in water								
0·3% (1900 ppm)	8·9	20	5·58±0·56^{ab}	5·38±0·51^{a}	0·52			
1·5% (9500 ppm)	11·2	20	4·59±0·67^{b}	4·32±0·62^{b}	1·51			
3·0% (18 000 ppm)	11·6	20	2·13±0·63^{c}	n.d.	3·97			

*Values shown are the means of data from two separate trials per experiment ± s.d. except for Experiment 4 (means from four separate trials). Means in each column for each experiment with no letters in common are significantly different ($P<0·05$).
†Seeds were rinsed with sterile tap water both before and after all treatments including the buffer and water controls.
‡TSA, trypticase soy broth; Petrifilm, *E. coli*/coliform Petrifilm.
§Log reductions based on populations (log cfu g^{-1}) of survivors determined by plating on TSA.
‖Values shown as % weight/volume and ppm free chlorine.
¶n.d. = none detected. The detection limit was 0·66 cfu g^{-1} for plating onto the Petrifilm *E. coli*/coliform count plates. The detection limit was 6·60 cfu g^{-1} for plating onto XLT-4 agar media.

among treatments with 2·0, 2·5 or 3·0% (w/v) solutions of $Ca(OCl)_2$ prepared in sterile KP buffer, pH 6·8 (11 000, 13 500, and 16 000 ppm free chlorine, repectively), or sterile tap water (14 000, 16 000 and 18 000 ppm free chlorine, respectively), for seed inoculated with *E. coli* O157:H7. Significant reductions (4·0–4·5 log cfu g^{-1}) in the populations of *E. coli* O157:H7 in relation to the buffer or sterile water controls were observed for the sterile tap water rinses plus treatment with buffered 2·5 or 3·0% (w/v) $Ca(OCl)_2$ solutions. A sharp drop-off in treatment efficacy occurred for 2·0% $Ca(OCl)_2$. A 10 min treatment with buffered 3·0% (w/v) of $Ca(OCl)_2$ along with the seed rinses also led to a significant reduction of *Salmonella* (3·9 log cfu g^{-1}) compared to the buffer plus rinses alone. Treatment with 2·5 and 3·0% (w/v) $Ca(OCl)_2$ led to a large increase in the percentage of injured bacterial cells present as evident by the lack of growth on selective agar media (*E. coli*/coliform Petrifilm and XLT-4). The extent of injury seen in this study for the pathogens after chlorine treatment of the inoculated seed was greater than that reported previously (Lang et al. 2000; Weissinger and Beuchat 2000). However, a high degree of injury was also reported after treatment with a variety of organic acids alone or in combination with a lower level (2 000 ppm) of free chlorine (Lang et al. 2000). Thus, for any sanitizer treatment examined for use on inoculated alfalfa seed, injured cells need to be accounted for.

Treatment with similar levels of $Ca(OCl)_2$ prepared in tap water plus the sterile tap water rinses were less effective in reducing the populations of *E. coli* O157:H7 on alfalfa seed (Table 2). The effectiveness of the unbuffered chlorine solutions did not appear to be due to the high pH (11·4–11·6) of the solutions based on a minimal effect of treating with 500 mM CAPS buffer, pH 11·0. Increasing the treatment time with 3·0% (w/v) unbuffered $Ca(OCl)_2$ from 10 to 20 min increased the log kill obtained for *E. coli* O157:H7 only by approximately 0·5 log units (4·0 log cfu g^{-1} compared to 3·5 $\log_{10}$ cfu g^{-1}).

Experiments were conducted to determine if reducing the treatment time from 10 to 5 min had a negative effect on efficacy. Results indicated that for buffered 3·0% (w/v) $Ca(OCl)_2$, the 5 and 10 min treatments were equivalent, but for unbuffered 3·0% (w/v) $Ca(OCl)_2$ the 10 min treatment was significantly more effective than the 5 min treatment (Table 3).

The log reductions obtained by treatment with high levels of chlorine were intermediate between published values from other laboratories for the two pathogens inoculated onto alfalfa seed. Lang et al. (2000) found that a 15 min treatment with unbuffered 20 000 ppm free chlorine led to a decrease of 6·9 log cfu g^{-1} for *E. coli* O157:H7. Using a considerably lower initial inoculum load on the seed, 3–10 min treatment times, and buffered 20 000 ppm free chlorine, Taormina and Beuchat (1999) reported an approximate 2·5 log cfu g^{-1} decrease for *E. coli* O157:H7 and Weissinger and Beuchat (2000) reported a 2 log cfu g^{-1} decrease in *Salmonella*. Variability in results among the various reports on the efficacy of chlorine treatments including the present study might be attributed to the differences in the alfalfa seed lots used (Beuchat et al. 2001) as well as to the methodology employed by the different labs. Among these differences in methodology is the initial inoculum level on the seed, the ratio of seed to volume of sanitizer, different treatment times and agitation methods, the use of buffered solutions, and the use of seed rinses before and after treatment. However, our study and all the studies cited above as well as that of Beuchat et al. (2001) are in agreement that treatment of laboratory-inoculated alfalfa seed with high levels of free chlorine does not totally eliminate the pathogens from the seed.

Addition of the surfactants Tween 80 (0·02% v/v), SDS (0·02% w/v) and a commercial chlorine potentiator (0·084%, v/v) to solutions containing unbuffered 3·0% (w/v) $Ca(OCl)_2$ did not increase the log kill for 10 min treatments of seed inoculated with *E. coli* O157:H7 (data not shown). The use of higher levels of surfactants may be beneficial, however, as Tween 80 (1%) used in combination with 1% (w/v) CaOH was recently reported to result in an additional 1 $\log_{10}$ cfu g^{-1} reduction of *Salmonella* on alfalfa seed compared to CaOH treatment alone (Weissinger and Beuchat 2000).

The effect of presoaking seed for 1 h after the initial seed rinses and before treatment with buffered 3·0% (w/v) $Ca(OCl)_2$ on treatment

Table 3. Comparison of the efficacy of 5 vs 10 min treatments with chlorine of alfalfa seed inoculated with *Escherichia coli* O157:H7

Treatment	Solution pH	Time (min)	Survivors ($\log_{10}$ cfu g^{-1})*	Log cfu g^{-1} reduction
Experiment 1:				
None	—	—	$5{\cdot}82 \pm 0{\cdot}31^{a}$	—
Buffer†	6·8	5	$3{\cdot}94 \pm 0{\cdot}24^{b}$	1·88
		10	$4{\cdot}15 \pm 0{\cdot}43^{b}$	1·67
3·0% $Ca(OCl)_2$	6·8	5	$1{\cdot}19 \pm 0{\cdot}33^{c}$	4·63
in buffer		10	$1{\cdot}21 \pm 0{\cdot}87^{c}$	4·61
Experiment 2:				
None	—	—	$5{\cdot}62 \pm 0{\cdot}01^{a}$	—
Sterile tap water	6·7	5	$4{\cdot}04 \pm 0{\cdot}09^{b}$	1·58
		10	$4{\cdot}02 \pm 0{\cdot}02^{b}$	1·60
3·0% $Ca(OCl)_2$		5	$3{\cdot}10 \pm 0{\cdot}30^{c}$	2·52
in water	11·6	10	$2{\cdot}03 \pm 0{\cdot}36^{d}$	3·59

*Values shown for Experiments 1 and 2 are the means of data from five and three separate experiments, respectively, calculated from data for plating on tryptic soy agar. Means for each experiment with no letters in common are significantly different ($P<0{\cdot}05$).
†Buffer was 500 mM potassium phosphate, pH 6·8.

efficacy for alfalfa seed inoculated with *E. coli* O157:H7 is shown in Table 4. The presoak step had little effect on the populations of bacteria removed by the buffer alone treatment (an increased reduction of 0·6 log cfu g^{-1}). A greater beneficial effect of the soaking step was obtained for the chlorine treatment (an increased log reduction of 1·8 log cfu g^{-1}). Unfortunately, the presoak step had a highly detrimental effect on germination. After 2 days of incubation, the values were 63% seed germinated for presoaked/chlorine-treated seed versus 90% for non-presoaked/buffer-treated seed. A shorter duration presoak (30 min) of inoculated alfalfa seed in water was reported to have a negligible effect on the efficacy of a treatment with 2 000 ppm free chlorine (Weissinger and Beuchat 2000).

Effectiveness of chlorine treatments on naturally contaminated seed

During 1999 there was a multi-state outbreak with at least 157 laboratory-confirmed cases of salmonellosis due to the consumption of contaminated alfalfa sprouts (Proctor et al. 2001). Isolates of *Salmonella* Muenchen from patients matched those obtained from alfalfa seed lot COA98.

Treatment of naturally contaminated alfalfa seed from lot COA98 with buffered 0·3% (w/v) $Ca(OCl)_2$ (1 800 ppm free chlorine) was not effective in eliminating *Salmonella* Muenchen. Of the six samples of treated seed, three were confirmed positive for *Salmonella* after sprouting (Table 5). Two of the three rinse water samples that were positive for *Salmonella* by immunoassay were confirmed to be positive by use of selective agar media. All three of the corresponding sprout homogenates were positive for *Salmonella*. The same number of germinated seed samples were confirmed positive for *Salmonella* for the buffer controls. At least one purified isolate per positive homogenate or rinse water sample was confirmed to be *Salmonella* by slide agglutination using commercial polyclonal antisera and biochemical tests.

In contrast, treatment of the naturally contaminated seed with buffered 3·0% $Ca(OCl)_2$ and unbuffered 3·0% (w/v) $Ca(OCl)_2$ eliminated the pathogen (Table 5). For seed treated with buffered 3·0% $Ca(OCl)_2$, all 12 sprout homogenates (Protocols A and B) as well as all six rinse water samples (Protocol B) were negative for the presence of the pathogen. Results for the buffer controls showed that six of the 12 sprout homogenates were positive for the pathogen (three by use of Protocol A and three by use of Protocol B). Two of the six rinse water

Table 4. Effect of addition of a presoak step on the ability of a chlorine treatment to eliminate *E. coli* O157:H7 from alfalfa seed

Treatment	Presoak step*	Survivors (log_{10} cfu g^{-1})†	Log (cfu g^{-1}) reduction
None	No	$6{\cdot}89 \pm 0{\cdot}87^{a}$	—
Buffer alone‡	No	$5{\cdot}90 \pm 0{\cdot}83^{ab}$	0·99
	Yes	$5{\cdot}32 \pm 0{\cdot}84^{bc}$	1·57
3·0% $Ca(OCl)_2$ in buffer,	No	$3{\cdot}90 \pm 1{\cdot}72^{c}$	2·99
(16 000 ppm free chlorine)	Yes	$2{\cdot}13 \pm 0{\cdot}47^{d}$	4·76

*Inoculated seed was soaked for 1 h in sterile tap water in between the initial sterile tap water rinse steps before a 10 min chlorine treatment was applied.
†Values shown are means of data for plating on tryptic soy agar from four separate experiments ± s.d. Means with no letters in common are significantly different ($P < 0{\cdot}05$).
‡Buffer was 500 mM potassium phosphate, pH 6·8.

Table 5. Ability of free chlorine treatments to eliminate *Salmonella* Muenchen from naturally contaminated alfalfa seed

	Number of positive tests/total tests			Total number of positive/total seed samples germinated
	Protocol A*	Protocol B		
Seed treatment	Homogenate	Homogenate	Rinse water	
(1) Buffered† 0·3% (w/v) $Ca(OCl)_2$, pH 6·8	n.d.‡	3/6	3/6 (2/6)§	3/6
Buffer alone	n.d.	1/6	4/6 (3/6)	3/6
(2) Buffered 3·0% (w/v) $Ca(OCl)_2$, pH 6·8	0/6	0/6	0/6	0/12
Buffer alone	3/6	3/6	2/6 (1/6)	6/12
(3) 3·0% (w/v) $Ca(OCl)_2$ in tap water, pH 11·6	n.d.	0/6	0/6	0/6
Sterile tap water alone	n.d.	2/6	2/6 (2/6)	3/6

*Protocol A consisted of testing sprout homogenates for *Salmonella* Muenchen by non-selective preenrichment, selective enrichment and plating on selective media. Protocol B consisted of testing sprout homogenates for *Salmonella* Muenchen as above as well as testing sprout rinse water using a commercial immunoassay kit.
†Buffer was 500 mM potassium phosphate, pH 6·8.
‡n.d., not determined.
§Numbers in parenthesis indicate the number of samples confirmed to be positive by plating on selective media and subsequent characterization of presumptive colonies of *Salmonella*.

samples tested using Protocol B were found to be positive by immunoassay, but only one of these two samples gave colonies typical of *Salmonella* after plating from the tubes of TSB+DNP+n onto selective agar media. Treatment with unbuffered 3·0% $Ca(OCl)_2$ was also effective as none of the six sprout homogenates or rinse water samples tested positive. For the corresponding buffer controls, two of the six sprout homogenates tested positive and two of the six rinse water samples tested positive. The two positive rinse water samples gave rise to typical colonies for *Salmonella* on selective agar media after plating from the tubes of TSB+DNP+n. At least one purified isolate per positive sample was confirmed to be *Salmonella* by slide agglutination and biochemical tests. For the data generated for treatment with 3·0% $Ca(OCl)_2$ with or without the buffer being present, statistical analysis indicated that the

values for the controls were statistically non-zero ($P<0{\cdot}05$). A total of 12 purified isolates found to be *Salmonella* based on typical colony formation on selective agar media, positive slide agglutination tests and biochemical tests were submitted for serotyping. Of these isolates, eight were typed as *Salmonella* Muenchen and four as *Salmonella* Bredeney. Contamination of alfalfa seed lots with more than one *Salmonella* serotype has been reported previously for at least four sprout-related outbreaks (Inami and Moler 1999, Inami et al. 2001, National Advisory Committee on Microbiological Criteria for Foods 1999).

For experiments in which both sprout homogenates and rinse water were tested for the presence of *Salmonella* Muenchen (Protocol B), neither method was consistently better than the other. Overall, there was agreement between the two sets of samples for 26 out of the 36 test results. There were four instances where the pathogen was detected in the sprout homogenates, but not in the corresponding rinse water samples. Conversely, there were three instances where the pathogen was detected in the rinse water samples, but not in the corresponding sprout homogenates. For the immunoprecipitate assay, out of the 11 total positive test results, eight test results were culture-confirmed as positive. This may indicate that, on occasion, native bacteria are present on alfalfa sprouts that give cross-reactions with the polyclonal antisera used in the assay kit. Stewart et al. (2001) tested a second enzyme immunoassay recommended for testing of spent irrigation water by the US FDA (1999) (Assurance Gold *Salmonella* EIA, Biocontrol Systems, Inc.) to examine sprout rinse water after germination of two naturally contaminated seed lots. They also reported some discrepancies between results from the rinse water testing and the corresponding sprout samples, but only at days 0 and 1. At days 2 and 3 of sprouting no discrepancies were noted. The results indicate that testing samples of both sprouts and sprout rinse water for pathogens would increase the sensitivity of sampling schemes under commercial conditions, but may not be feasible due to cost considerations.

The inability of relatively low levels of free chlorine (1800–2000 ppm) to eliminate *Salmonella* serotypes from the naturally contaminated seed lot COA98 agrees with our results discussed above with laboratory-inoculated seed as well as previous studies using laboratory-inoculated alfalfa seed (Beuchat 1997, Weissinger and Beuchat 2000). In contrast, studies by Suslow and associates (Suslow et al. 2001) found that treatment of an alfalfa seed lot naturally contaminated with *Salmonella* Mbandaka with unbuffered 2 000 ppm or buffered and unbuffered 20 000 ppm free chlorine from $Ca(OCl)_2$ for 30 min or 10–15 min, respectively, eliminated the pathogen. Stewart et al. (2001) recently reported that a 10 min treatment with unbuffered 20 000 ppm free chlorine from $Ca(OCl)_2$ did not eliminate *Salmonella* Muenchen or *Salmonella* Mbandaka from the two naturally contaminated alfalfa seed lots. In that study, seed was mixed with the chlorine solutions only at the beginning of the treatment period. The differing results obtained using laboratory vs naturally contaminated alfalfa seed as well as obtained for the naturally contaminated seed lots might be due to a variety of factors including differences in the pathogen load on the seed, the physical condition of the seed (Charkowski et al. 2001, Holliday et al. 2001) and location of the pathogens on the seed, treatment time and method and extent of agitation during treatment. The inability to eliminate bacterial human pathogens from laboratory-inoculated seed might be due to the typical inoculation method used (submerging seed in an aqueous bacterial suspension). This inoculation method might result in the deposition of bacterial cells deep into natural openings such as the hilum or micropyle or cracks in the seed coat where they are protected from aqueous sanitizers. Natural routes of contamination in the field may not result in bacterial pathogens being present in such inaccessible sites.

The increased efficacy of the seed treatment with unbuffered 3·0% $Ca(OCl)_2$ (18 000 ppm of free chlorine, pH 11·6) over that for buffered 0·3% $Ca(OCl)_2$ (1 800 ppm free chlorine, pH 6·8) demonstrates that the amount of hypochlorous acid present was not the sole determining factor for efficacy of the chlorine treatment. Hypochlorous acid is reported to have 80 fold greater antibacterial activity than

does the hypochlorite ion (Dychala 1991). At a pH of 11·6 and a temperature of 20°C, approximately 0·03% of the chlorine is present as hypochlorous acid whereas at a pH of 6·8 and a temperature of 20°C the value is approximately 75%. Thus, an 18 000 ppm free chlorine solution at pH 11·6 will contain approximately 5 ppm of hypochlorous acid and 17 995 ppm of hypochlorite ion (equivalent in antibacterial activity to 225 ppm of hypochlorous acid). A 1800 ppm solution of free chlorine at pH 6·8 will have approximately 1350 ppm of hypochlorous acid and 450 ppm of hypochlorite ion. Thus, the buffered solution containing 1800 ppm free chlorine would be expected to be more antibacterial than the unbuffered solution containing 18 000 ppm free chlorine. Possibly, the greater effectiveness of the unbuffered solution of 18 000 ppm of free chlorine is due to the release of a greater amount of chlorine gas during seed treatment (Adams et al. 1989). Chlorine in the gaseous state may be better able to reach bacteria in protected locations on the seed.

A limited number of volatile antimicrobial compounds have been tested for sanitizing seed. Treatment of laboratory-inoculated mung bean seed with acetic acid vapor for 12 h at 45°C eliminated *Salmonella*, *E. coli* O157:H7 and *Listeria monocytogenes* without reducing seed germination (Delaquis et al. 1999). Treatment of laboratory-inoculated alfalfa seed with allyl isothiocyanate (50 µl in a 950-cm^3 container) for 24 h at 37 or 47°C totally eliminated *E. coli* O157:H7 if the seed was wetted before treatment. Unfortunately, the treatment led to an unacceptable reduction in seed viability (Park et al. 2000). Most recently, fumigation of laboratory-inoculated alfalfa and mung bean seed with ammonia was reported to result in a 2–log and 5–6 log cfu g^{-1} reduction of pathogens (*E. coli* O157:H7 and *Salmonella* Typhimurium), respectively (Himathongkham et al. 2001).

In conclusion, elimination of bacterial human pathogens from laboratory-inoculated alfalfa seed appears to be more problematic than elimination of pathogens from naturally contaminated seed in the laboratory. This may be due to differing populations and/or location of the contaminants. Treatment of alfalfa seed with high levels of free chlorine from $Ca(OCl)_2$ by sprout growers as recommended by the US FDA should lead to a reduction in the number of alfalfa sprout-related outbreaks of foodborne illness. However, as among laboratories, it is expected that individual growers will differ in the methodology used for applying a chlorine treatment to the seed and these differences along with several other factors will affect treatment efficacy. This is borne out by a recent report on the 1999 *Salmonella* Muenchen related outbreak which stated that at least some of the growers implicated reported treating their seed with 20 000 ppm for 15 min (Proctor et al. 2001). Thus, sprout growers should test spent irrigation water for pathogens in addition to the seed sanitization step as recommended by the US FDA (1999).

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